Assay Name: Antibody-Dependent Receptor Internalization Assay

Assay ID: Celigo_02_0015

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Experiment: Antibody-Dependent Receptor Internalization Assay

Purpose	To measure the level of receptor internalization induced by known antibody
Current Method(s)	Flow Cytometry
Target Cell Type	GFP expressing HT-293 cell line
Experiment Plan	Compare receptor internalization level between a positive control antibody and a negative control antibody at different concentrations
Hypothesis	Results will show increase in internalization correlating to increased fluorescent signals from the positive control group.

Celigo Setup

Plate Type	Greiner 96-well, black wall clear bottom, Cat # 655090
Scan Channels	Bright field, Red, Green
Resolution	1 µm/pixel
Scan Area	Whole well
Analysis Method	Target 1 + 2 + Mask
Scan Frequency	Hourly, up to 5 hours
Scan Time	~9 min

Assay Protocol and Plate Setup

Goal

To measure the level of receptor internalization induced by known antibody.

Protocol

Cell preparation

1. Collected target cells and seeded at 10,000 cells/well into each well using the plate map below
    a. Wells labled with Media are control wells with cells and media only
    b. N1-N5 are serial dilution of the negative antibody, where N5 is the highest concentration
    c. P1-P5 are serial dilution of the positive antibody, where P5 is the highest concentration
2. Pipetted the positive and negative antibodies at different concentrations following the plate map below
3. The antibodies were pre-labeled with a pH-sensitive dye using a kit from Promega and bind to receptors on the target cells
4. The plates were then scanned using the Celigo at T = 0, 1, 2, 3, 4 and 5 hours

Drug	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		Media	N 1	N 2	N 3	N 4	N 5					
C		Media	P 1	P 2	P 3	P 4	P 5					
D		Media	N 1	N 2	N 3	N 4	N 5					
E		Media	P 1	P 2	P 3	P 4	P 5					
F												
G												
H												

Data Collection

1. After adding the cells and antibodies, the plate was scanned in Celigo using Target 1 + 2 + Mask application for t = 0 h
2. Repeat the scanning for t = 1, 2, 3, 4, and 5 h

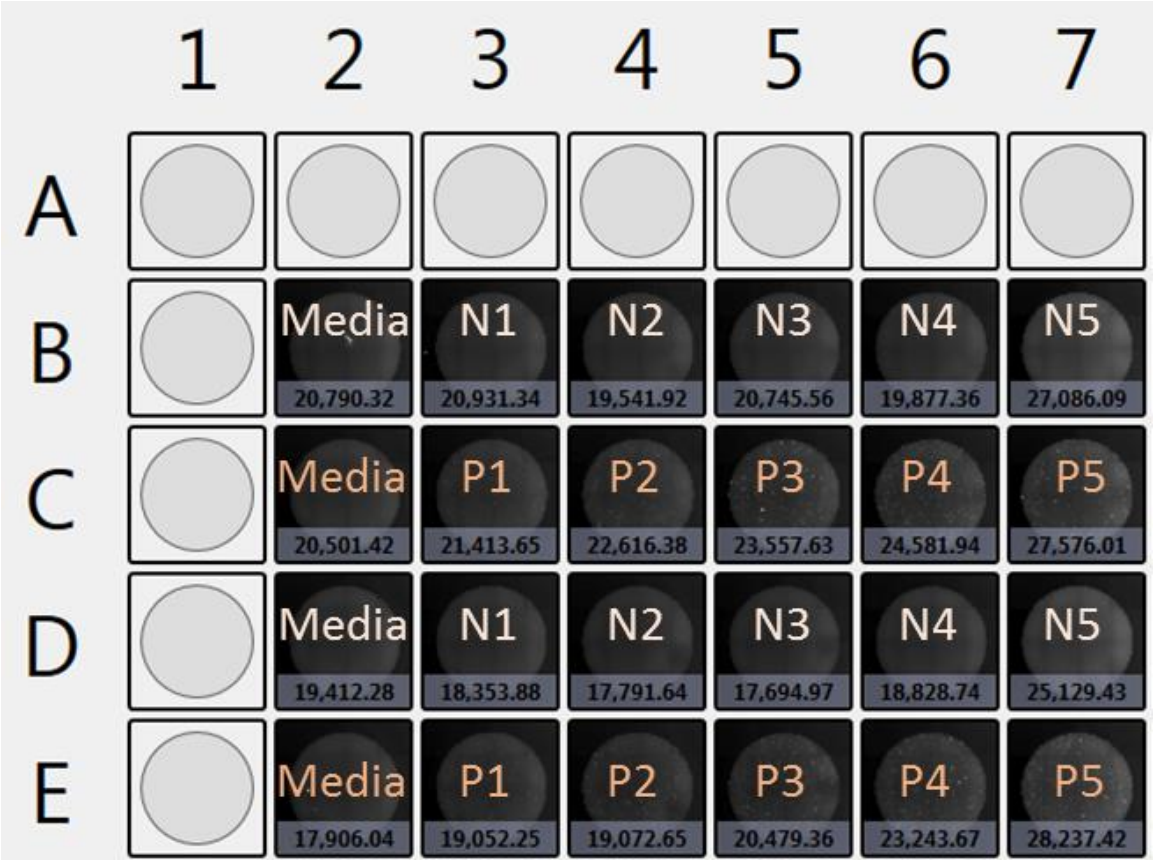
Data Analysis

- The images at each time point were analyzed to count the total number of GFP positive cells
- Next, the red fluorescent intensity was measured from within the cells to determine the level of receptor internalization compared to the negative controls

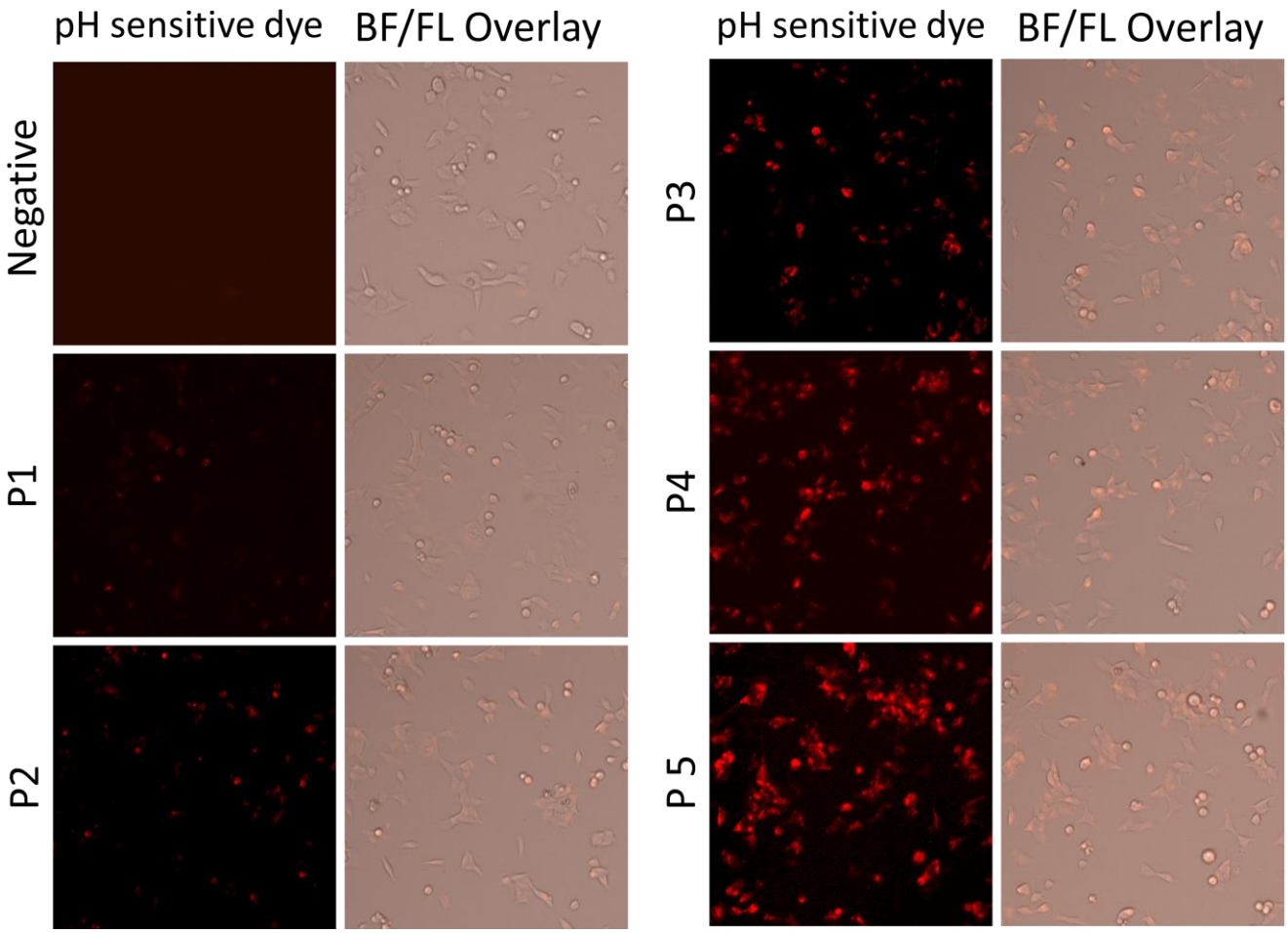
Results

1. Receptor Internalization images using Celigo Imaging Cytometer

- Celigo was used to capture bright field and fluorescent whole-well images on 96-well plates
- It required ~9 min/plate for image acquisition and fluorescent data analysis
- Whole plate overview can be viewed to quickly assess the receptor internalization results (See figure below)
    - In this example, we showed a plate view of HT-293 at 5 hour on the Celigo software to provide a quick at-a-glance result of the receptor internalization
    - The values shown in each well are the total or integrated fluorescent intensities

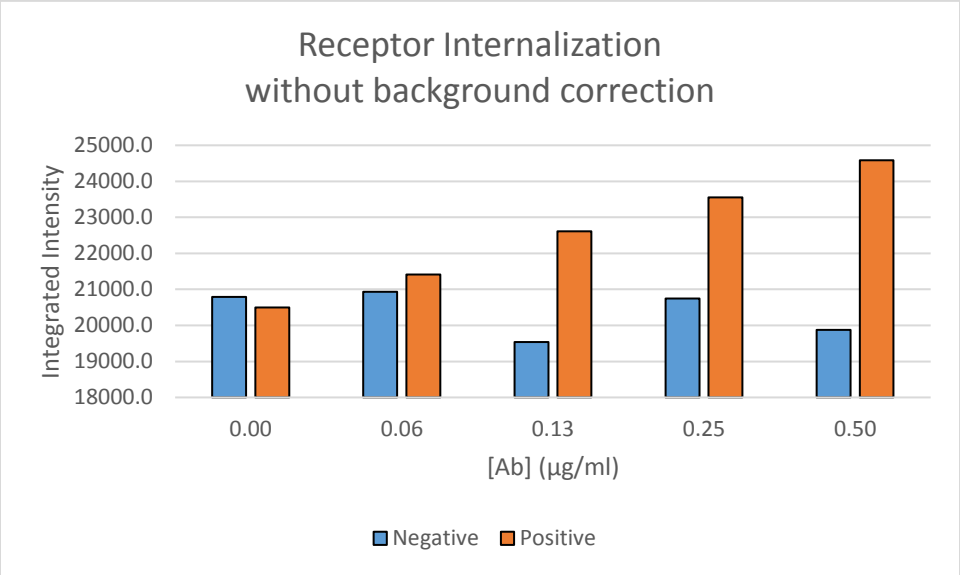


- Each well can be further zoomed-in to view images of individual wells and cell populations in the wells, which allows visual confirmation (See figure below)
    - In this example, we showed HT-293, zoomed-in view at 5 hour and different Antibody treatment concentrations
    - It is clear that the red fluorescence increased as the concentration of positive antibody increased

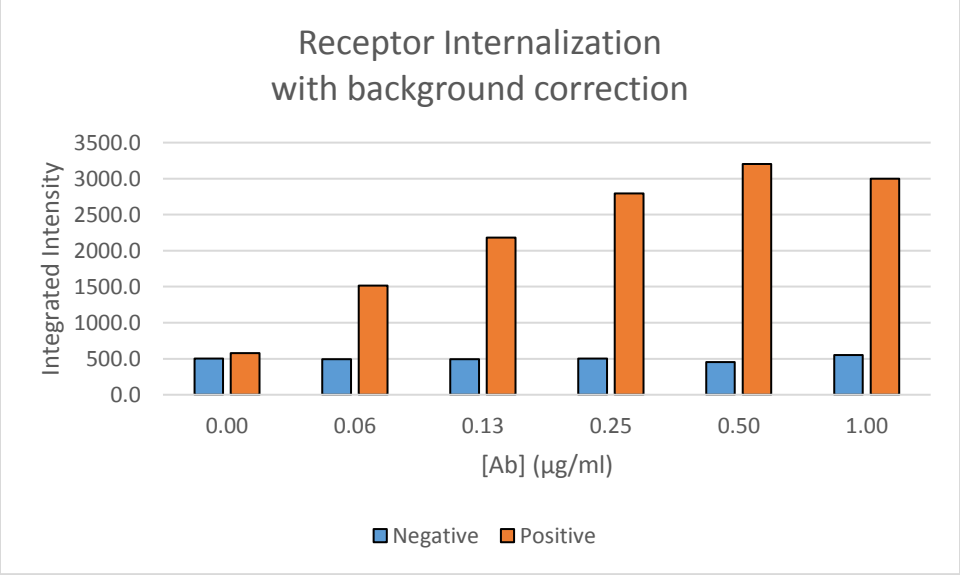


2. Antibody-dependent receptor internalization results

- The receptor internalization analysis measured the total fluorescent intensity in the cell population treated with different Antibody concentrations
- The Negative Antibody showed no fluorescent signals, which was observed in the images as well
- By measuring the total fluorescent intensities in the cells, we saw an increase in signal as the concentration of positive Antibody increased



- In addition, Celigo software was able to perform background correction to improve signal-to-background ratio with resulting data showing below



Conclusion

- The results showed a clear difference between negative antibody and positive antibody in internalization
- A dose response was observed for positive antibody by measuring average total fluorescent intensity
- By using the background correction function on Celigo, the background can be removed to increase the fluorescent intensities